Fe-S Synthesis in Algae Mitochondria

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Iron and sulfur are two essential elements for all organisms. These elements form the Fe-S clusters that are present as cofactors in numerous proteins and protein complexes related to key processes in cells, such as respiration and photosynthesis, and participate in numerous enzymatic reactions.

Keywords: mitochondria ; algae ; iron-sulfur

1. Introduction

Iron (Fe) is an essential micronutrient for all aerobic organisms. However, it is highly toxic in the free state and can cause oxidative stress and damage to cellular macromolecules. For this reason, organisms developed mechanisms to regulate the content of Fe, responding to the deficiency or increase of the metal. Fe is present in numerous proteins that participate in energy metabolism, such as those that are involved in the mitochondrial and chloroplastic electron transport chains [1].

Both Fe and S are two compounds that are important for the synthesis of Fe-S groups and ferrosulfoproteins. These groups are inorganic cofactors that are present in numerous proteins that participate in different metabolic pathways such as photosynthesis and respiration, previously mentioned, but also in the regulation of gene expression, protein translation, maintenance of DNA integrity, and in metabolic pathways related to the assimilation of nitrogen, sulfur and iron, and amino acid metabolism [2][3][4].

Although there are numerous works that characterized the function and regulation of genes and proteins that participate in the production of Fe-S groups in bacteria, yeasts, and humans, little is known about the occurrence and function of these genes in photosynthetic organisms, especially in algae.

2. Fe and S Uptake and Metabolism in Algae

Fe-S clusters are among the most ancient cofactors used by living organisms. Life originated in water, where originally its constituent elements were abundant and easily accessible. However, with the evolution of photosynthetic organisms, the atmosphere and the oceans filled with oxygen, and the bioavailability of the elements radically changed. Chlorophytes have to incorporate the substrates for the synthesis of Fe-S centers, Fe and S, from the surrounding medium. In primeval times, Fe was present as the soluble ferrous (+2) ion. However, in oxygenic environments, Fe is oxidized to ferric (+3) ion, which forms insoluble oxides and hydroxides [5]. Land plants developed two different strategies to solubilize the metal: reduction and chelation. Both are energy expensive, but it's the price to continue taking advantage of the chemical properties of Fe for biological reactions. Most work regarding Fe metabolism in chlorophytes was done with the model algae *Chlamydomonas reinhardtii* because it is easy to grow in a synthetic defined medium, and its genome has retained genes from the last common ancestor from both the plant and animal lineages. By homology searches in genomic and transcriptomic databases, particularly from algae grown in metal deficient conditions, with known yeast, animal, and plant transporters as baits, the repertoire of Fe and other metal transporters in *Chlamydomonas* and other algae has been analyzed [6]. *C. reinhardtii* incorporate Fe by a reduction-based strategy. Insoluble ferric oxides and other salts are solubilized by the activity of ferric reductases on the plasma membrane. *Chlamydomonas* has one gene of the NOX family of ferric reductases, *FRE1*, orthologous to the *Fre1/Fre2* and *FRO2* genes involved in Fe uptake in yeast and *Arabidopsis thaliana*, respectively. FRE1 is localized to the plasma membrane and is induced by Fe deficiency, coordinately with FEA1, FEA2, FOX1, and FTR1 [7]. FOX1 and FTR1 are similar in molecular functions to the Fet3p/Ftr1p pair in yeast, and perform ferroxidation and high affinity Fe^{3+} uptake in *Chlamydomonas*, respectively [6][8]. FOX1 is a multicopper oxidase with the highest similarity to human ceruloplasmin and hephaestin, and this fact explains the copper-dependency for Fe assimilation in this alga, like in yeast [9]. The permeases like FTR1 are abundant in algal genomes and are thought to channel ferric ions to the cytoplasm, intimately associated with the multicopper oxidases; however, some chlorophytes do not express these proteins, nor they have the corresponding genes (see below). *Chlamydomonas* cells have an extracellular space between the wall and the plasma membrane that houses proteins involved in nutrient assimilation [10]. FEA are algal-specific proteins secreted to this periplasmic space. Although the exact biochemical and molecular role of these proteins remains to be determined, they are the major secreted proteins during Fe deficiency and are most probably involved in Fe assimilation. *Chlamydomonas* lacking cell wall secrete FEA proteins to the medium and are more sensitive to Fe deficiency compared to algae with cell walls that retain FEA proteins [7].

Recently *Ostreococcus tauri* has been proposed as a new model green alga for Fe metabolism studies [11]. This alga grows in open oceans where Fe scarcity is more prevalent than in *C. reinhardtii* growing niches. The most striking characteristic in *O. tauri* Fe uptake is that it does not seem to involve Fe reduction, because it lacks the classical components. *O. tauri*, and other green algae like *Ostreococcus lucimarinus* or *Micromonas* sp., do not have genes for the *FOX1*/*FTR1* pair [6], and although they encode ferric reductases, they are not induced by Fe deficiency [11]. Additionally, there is no clear connection between copper and Fe metabolism, and, instead, zinc seems to play an important role in regulating iron uptake [11]. Several other genes coding for potential Fe uptake transporters have been detected in *O. tauri*, including one gene from the ZIP family, related to the *Arabidopsis* IRT1 high affinity Fe uptake transporter, and a gene from the algal FEA family, which seems to include potential transmembrane and Fe binding motifs [11].

Once iron enters the algal cell, it must be trafficked to the different organelles, and when in excess, transported to sites of storage. Major sinks for Fe are chloroplasts and mitochondria. However, rather little is known about transport of Fe across these membranes. The mitochondrial solute carrier (MSC) family is a large family of intracellular transporters involved in the transport of multiple substrates. Mrs3p/Mrs4p from yeast belong to this family and are involved in Fe transport across inner mitochondrial membrane [12]. MITs from rice and *Arabidopsis* are also members of the same family and were recently related to Fe traffic into the mitochondria [13][14]. A protein similarity network of the MSC family led to the identification of a cluster of proteins that includes Mrs3p/4p, plant MITs and predicted iron transporters from algae [14].

Sulfur, the second element in Fe-S clusters, and an essential macronutrient present in proteins, lipids, carbohydrates, and several metabolites, has also suffered a great change in its most abundant chemical form in the environment. In ancient oceans, it was mainly present as sulfide (S^{2-}), while with the advent of oxygenic photosynthesis, it was oxidized to sulfate (SO_4^{2-}). Primary producers have to invest energy in its reduction to sulfide in order to be able to incorporate it to biomolecules. The sulfate anion (SO_4^{2-}) is the preferred source of S, but *Chlamydomonas* also has the ability to acquire sulfur from organic compounds. This is achieved by the secretion of two arysulfatases (ARS) to the periplasmic space, capable of cleaving sulfate anions from esterified organic sulfate [15][16]. The high affinity sulfate transporters SULTR2 (plant type H^+/SO_4^{2-} cotransporter) and SLT1 and SLT2 (bacterial and animal type Na^+/SO_4^{2-} cotransporters) on plasma membrane mediate SO_4^{2-} uptake [17]. Once inside the cell, SO_4^{2-} must be reduced in order to be incorporated in biomolecules. But because SO_4^{2-} is a relatively inert molecule, the first step prior to assimilation is the activation by the enzyme ATP sulfurylase to render adenosine phosphosulfate (APS). In the pathway leading to reduction, the S of APS is reduced to sulfite by APS reductase, and sulfite is further reduced to sulfide by sulfite reductase. Finally, sulfide is incorporated into the amino acid skeleton of O-acetylserine (OAS) by OAS thiollyase to form cysteine [18], which could be further transformed into methionine, the methyl donor S-adenosylmethionine (AdoMet), and the antioxidant glutathione [19]. The assimilation process is essentially similar to that of *Arabidopsis*, and in all photosynthetic organisms, sulfate reduction occurs in the plastids, but ATP sulfurylases are also present in the cytosol, and plant ATP sulfurylases have a different evolutionary origin than those from green algae [20]. Cysteine, the source of S for the ISC Fe-S cluster biosynthesis, can be synthesized in the cytosol, plastids, and mitochondria in nearly all plant species. Current studies suggest a scenario in an Arabidopsis leaf where chloroplasts generate sulfide via reductive sulfate assimilation, the mitochondria provide the bulk of OAS, and the cytosol produces most of the cysteine [21]. It is unknown where the main synthesis of cysteine occurs in green algae, but *C. reinhardtii*, *Coccomyxa subellipsoidea* and *Volvox carteri* genomes contain several genes coding for OAS thiollyases [22].

Studies in *Chlamydomonas* have shown that transcript levels and activities of transporters and enzymes participating in the S assimilation pathway, such as ARS, SULTR2, SLT1, SLT2, ATP sulfurylase, APS reductase, and OAS thiollyase, increase under S deficiency conditions, and are highly regulated by the demand for reduced sulfur and by environmental conditions [23].

3. Synthesis of Fe-S Clusters

The most common and simplest forms of Fe/S clusters are of the (2Fe-2S) and (4Fe-4S) type, but also (3Fe-4S) forms or more complex clusters containing additional heavy metal ions are known [24][25]. Specifically, the iron-sulfur group biogenesis pathways include several proteins involved in apoprotein maturation in different cellular compartments [26].

Aerobic eukaryotic organisms depend on mitochondria to synthesize Fe-S groups, while photosynthetic aerobic organisms also synthesize Fe-S groups in chloroplasts [4][27]. Although mitochondria can assemble their own Fe-S proteins, they play a crucial role in the biogenesis of cytosolic and nuclear ferrosulfoproteins [26][27].

It was described in plants, by studies carried out mainly in *A. thaliana*, that there are three metabolic pathways for the assembly of Fe-S groups: (i) the SUF pathway (mobilization of sulfur) in chloroplasts, (ii) the CIA pathway of assembly of Fe-S groups in cytosol, and (iii) the ISC pathway, mitochondrial iron-sulfur cluster [3][27]. The SUF and ISC machines carry out the synthesis of Fe-S groups in three basic stages. In the first stage, S is obtained from the reaction catalyzed by a cysteine desulfurase, NFS, and combined with Fe on a scaffold protein for de novo synthesis of (2Fe-2S) groups. In a second stage, the Fe-S group is released from the scaffold with the aid of chaperones and co-chaperones, and bound by a transfer protein. At this point, the (2Fe-2S) group can be inserted into apoproteins, trafficked to the late-acting machinery

for (4Fe-4S) group synthesis, or, in the ISC pathway, used for export of a yet unknown sulfur-containing species to be utilized by the CIA system. The third stage is less known and comprises the conversion of (2Fe-2S) into (4Fe-4S) groups, and the insertion into apoproteins by dedicated targeting factors [27][28].

There are numerous works on the characterization of the synthesis pathway of Fe-S groups in mitochondria mediated by the ISC complex [29]. It has been reported that this complex is a heterodecamer composed of five proteins, NFS1, the scaffold protein ISCU, ISD11, an acyl-carrier protein (ACP), and frataxin [29].

Nfs1 is a pyridoxal phosphate-dependent mitochondrial cysteine desulfurase. This protein produces S from alanine [30]. It was shown that in plants, specifically in Arabidopsis, there are two isoforms, AtNFS1, located in mitochondria and AtNFS2, with chloroplastic localization [3][31][32][33]. ISD11 is a member of the LYR protein family, which lacks orthologs in prokaryotes but is highly conserved in yeast, humans, and plants [34][35][36], while IscU is a scaffold protein homologous to ISU from plants [37]. Three genes encoding ISU1-3 were found in Arabidopsis (At4g22220, At3g01020, At4g04080) [31][38][39], while ACP is an acyl group carrier protein, homologous to *Arabidopsis* AtACP1-3 [40]. Finally, frataxin is a highly conserved protein from prokaryotes to eukaryotes [41][42]. This protein has been involved in various processes such as Fe homeostasis, respiration, heme metabolism, oxidative stress, and transfer of persulfide groups [43][44][45][46][47][48][49].

3.1. Cysteine Desulfurases

As mentioned above, the first step in the synthesis of Fe-S groups in mitochondria and chloroplasts is catalyzed by a cysteine desulfurase [50]. These enzymes are found in the three kingdoms of life, representing a conserved group of proteins with several essential functions, such as the formation of Fe-S groups and the synthesis of biotin, thiamine, molybdenum cofactor, thionucleosides in tRNAs, and lipoic acid [50][51][52][53].

Cysteine desulfurases belong to the class V group of aminotransferases and are classified into two main classes (I and II) based on their sequence similarity [50][54]. A particularly recognized structural difference between type I and type II is the catalytic loop, which contains a nucleophilic cysteine. This catalytic loop of type I cysteine desulfurases is longer than that of type II enzymes and, therefore, it is considered to be structurally more flexible than that of type II enzymes [55][56][57].

Class I of cysteine desulfurases contains the consensus sequence SSGSACTS and group II, the sequence RXGHHCA. In general, the group I contains the enzymes with sequence similarity to NifS and Isc and those of class II, those enzymes related to SufS and CsdA [50]. The resolution of the crystal structures of the IscS cysteine desulfurases of *Escherichia coli* (EcIscS) and *Thermotoga maritima* (TmNifS), showed that these proteins form homodimers of around 90 kDa, also showing high structural similarity [55][58]. Each monomer is composed of a major domain in which the PLP cofactor is bound to a conserved lysine, and a minor domain that houses the active site cysteine [55][58][59].

The first cysteine desulfurases were described in the nitrogen-fixing bacteria *Azotobacter vinelandii*, called IscS and NifS. IscS is the cysteine desulfurase of the ISC system for the synthesis of Fe-S centers, while NifS is exclusively involved in the maturation of the enzyme nitrogenase [50][60]. In bacteria like *E. coli*, three genes were found that code for cysteine desulfurases: IscS, SufS (CsdB) and CsdA (CSD) [50]. On the other hand, in humans and in *S. cerevisiae*, there is a single gene that codes for a type I cysteine desulfurase called Nfs1 [61][62]. In humans, there are two isoforms of Nfs1 located in the cytosol and mitochondria, generated by the differential use of codons at the beginning of translation [61]. In yeast, due to differential cleavage of the signal peptide, Nfs1 is mainly localized in mitochondria but is also found in the cytosol and nucleus [63]. The deficiency of Nfs1 in both organisms produces a decrease in the activity of Fe-S proteins generating serious problems in growth [62][64].

Few cysteine desulfurases have been described in higher plants, such as those from *A. thaliana* and *Glycine max* [65][66]. In *Arabidopsis*, the presence of AtNFS1, located in mitochondria, and AtNFS2, present in chloroplasts was described [31][33][66][67][68]; while in *G. max* the presence of four cysteine desulfurases was reported [65]. The analysis of the amino acid sequences of AtNFS1 showed that this protein belongs to class I of cysteine desulfurases [33][68]. Homology modeling studies showed that AtNFS1 exhibits the characteristic folding of class I cysteine desulfurases such as IscS from *E. coli* [32]. AtNFS1 and AtNFS2 are essential for the plant since homozygous mutants are lethal, and the reduction in the abundance of these proteins produces severe consequences in development. Furthermore, there is an additional cysteine desulfurase called ABA3 involved in the formation of the molybdenum cofactor in the cytosol, which is not related to the biosynthesis of Fe-S and groups [69].

Currently, the genes and proteins that would participate in the synthesis of Fe-S groups in algae have not been identified or characterized. Thus, we performed a bioinformatic search for sequence identity for NFS1 and NFS2 in Chlorophytes using the Uniprot Database [70] and the *A. thaliana* sequences as a query. The search results showed a total of eighteen sequences for NFS1 and ten sequences for NFS2 in green algae. NFS1 homologs present a percentage identity between 62.2 and 70.6% with respect to AtNFS1, with a sequence length between 406 to 488 amino acid residues (Table 1). These sequences were analyzed in order to verify the conservation of critical amino acids for the function of the enzymes. For NFS1 homologs, the residues belonging to the active site of the enzyme are almost completely conserved (T124, H152, K153, N203, E204, K245 and Y386, according to the numbering of AtNFS1) as well as the catalytic residue Cys 377 and R403, involved in substrate binding. The active site of AtNFS2 has also almost all conserved residues belonging to the active site (T144, H172, H173, N224, V225, Q252, K275), a catalytic cysteine at position 418 (C418) and an arginine that

binds the substrate (R433, all positions according AtNFS2 numbering). The exceptions are those enzymes belonging to *Chlorella variabilis* and *Chlamydomonas eustigma*, where V225 is replaced by a methionine, and in the protein from *Monoraphidium neglectum*, where we found a threonine in that position.

Table 1. Orthologs proteins involved in Fe-S cluster synthesis in mitochondria of Chlorophytes.

ISC Proteins	NFS	ISU	NFU	ISD11	HSCA	HSCB	ACP
Function	**Cysteine desulfurase**	**Scaffold protein**	**Scaffold protein**	**Regulatory protein**	**Chaperone**	**Co-chaperone**	**Regulat protei**
***Arabidopsis thaliana*genes**	**At5g65720.1**	**At4g22220**	**At3g20970 (NFU4) At1g51390 (NFU5)**	**At5g61220**	**At4g37910 (HSCA1) At5g09590 (HSCA2)**	**At5g06410**	**At2g44 (mtACI At1g65 (mtACI At5g47 (mtACI**
UniProt	**O49543-1**	**O49627-1**	**Q9LIG6-1 Q9C8J2**	**Q8L9E3-1**	**Q8GUM2 Q9LDZ0**	**Q8L7K4**	**P5366 O808 Q9FG.**
			Identified Protein Sequences				
			Organism				
Auxenochlorella protothecoides	**A0A087SBH3 (c)**	**RMZ55380.1 (c)**	**A0A087SIP0**		**A0A087SND2**	**XP_011400182**	**A0A087S**
Bathycoccus prasinos	**K8FF08 (p)**	**XP_007511837.1**	**K8F7J7**		**K8F1V7**	**XP_007510865**	**K8FE**
Chlamydomonas eustigma		**GAX73632.1**	**A0A250XNZ5**		**A0A250XIU3**	**GAX75929**	**A0A250W**
Chlamydomonas reinhardtii	**PNW80545.1 PNW87417.1**	**XP_001693712.1**	**A0A2K3D318 A0A2K3D340**	**A8IZ28**	**A8IZU0 (c)**	**A8IK66**	**Q6UK**
Chlorella sorokiniana	**A0A2P6U3X4**	**PRW56423.1**	**A0A2P6TLS6**	**A0A2P6U0I2**	**A0A2P6TM18**	**A0A2P6TMK0 (c)**	**A0A2P6**
Chlorella variabilis	**XP_005843562.1 (c) XP_005848297.1 (c)**	**XP_005850086.1 (c)**	**E1ZCG7 (c)**		**E1ZMD2**	**XP_005845092 (c)**	**E1Z6**
Chloropicon primus	**A0A5B8MHP9**	**QDZ20066.1**		**A0A5B8MCM2**	**A0A5B8MHC3**	**QDZ23877**	
Coccomyxa subellipsoidea	**I0Z0B3 (c)**	**XP_005649671.1**	**I0YUZ0 (c)**	**I0Z768**	**I0YWN6**		**I0YSD**
Dunaniella salina		**KAF5838253 KAF5839176 (c)**			**KAF5837283.1 KAF5830450.1 KAF5832491.1**	**KAF5832703.1**	
Gonium pectorale		**KXZ49215.1**			**A0A150GMI8**	**KXZ55931 (c)**	**A0A150G**
Haematococcus lacustris		**GFH16011.1**		**A0A699ZQI7**		**GFH07090 (c)**	
***Helicosporidium*sp.**		**KDD73008.1 (c)**			**A0A059LST8**		**A0A059L**
Micractinium conductrix	**A0A2P6V8I3**	**PSC71357.1 (c)**	**A0A2P6V9W1**	**A0A2P6VEW0**	**A0A2P6VHZ4**	**PSC73872**	**A0A2P6**
Micromonas commoda	**XP_002506217.1 (c) XP_002501646.1 (c)**	**XP_002502856.1 XP_002507274.1 (c)**	**C1EHF7**	**C1E8J2**	**C1EGS6**	**XP_002505982.1**	**C1E2**
Micromonas pusilla		**XP_003055300.1**		**C1MZU9**	**C1MP69**	**XP_003057174**	**C1N9**
Monoraphidium neglectum	**A0A0D2M031 (c)**	**XP_013897551.1**				**XP_013898922**	**A0A0D2**
Ostreococcus lucimarinus		**XP_001415848.1**	**A4RUX0**	**A4RYZ0**	**A4RWG3**	**XP_001419576**	**A4S9**
Ostreococcus tauri	**XP_003078141.1 XP_003081355.2**	**XP_022838219.1 0US42676.1**	**Q01C69**	**A0A090M6V6**	**Q01AH9**	**XP_022839639**	**Q00SI**
Pycnococcus provasolii		**GHP08054.1**					
Raphidocelis subcapitata	**A0A2V0PAY6**	**GBF95838.1**		**A0A2V0PLS6**	**A0A2V0PIR9**	**GBF88744**	**A0A2V0**
***Scenedesmus*sp.NREL46B-D3**		**KAF6244147.1**				**KAF6263471**	
***Scenedesmus*sp.PABB004**		**KAF8055824.1**				**KAF8065471**	

Tetrabaena socialis		**PNH03155.1**	**A0A2J8AFK7**	**A0A2J8A4Z1**			**A0A2J8**
Tetradesmus obliquus	**A0A383VZF3**		**A0A383V5U1**	**A0A383WNF2**	**A0A383W6Z2**	**A0A383WKP1**	**A0A383V**
***Trebouxia*sp. A1–2**		**KAA6417420**				**KAA6420365 (c)**	
***Volvox carteri*f. nagariensis**	**XP_002951960.1**		**D8TNE6**	**D8TVN4**	**D8TMR1 (c)**	**XP_002948275**	**D8U1**

Ref: (c) indicates cytosolic localization, (p) indicates chloroplastic localization, (p,m) indicates dual localization in plastids and mitochondria. All other proteins where no localization is indicated showed a predicted mitochondrial localization.

The presence of multiple cysteine desulfurases was previously described in other organisms such as *Bacillus subtilis* and other gram-positive bacteria. However, this had not been described in photosynthetic organisms [71]. We found two putative NFS1 proteins in *C. reinhardtii*, *C. variabilis*, *O. tauri* and *M. commoda*, whereas one NFS1 was identified in all the other algae species (Table 1). The presence of multiple NFSs in these algae could be a strategy to distribute sulfur towards different metabolic pathways. It would also be of great interest to study the possible participation and functions of these multiple mitochondrial cysteine desulfurases in the synthesis pathways of Fe-S groups in the four algae species mentioned above. On the other hand, since one of the *B. subtilis* cysteine desulfurases was reported to have reactivity towards cysteine in the presence of a sulfide transferase (SufU), it is possible that some of these desulfurases also interact with other proteins to fulfill their biological function.

To analyze the cell location, we use the Deep-Loc1.0 server [72]. The proteins found in *C. sorokiniana*, *M. conductrix*, *T. obliquus*, *V. carteri* f. nagariensis, *O. tauri* (*2*), *R. subcapitata*, *C. primus*, *M. commoda* (*2*), *C. reinhardtii* (*2*) and *C. variabilis* (*2*), would present mitochondrial localization while in *B. prasinos* the protein would be located in plastids. The analysis for the sequences of *A. protothecoides*, *C. subellipsoidea*, *M. commoda*, *M. neglectum*, and *M. pusilla* showed a cytoplasm localization.

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